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Caspase-2 Mediates Site-Specific Retinal Ganglion Cell Death After Blunt Ocular Injury

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Purpose. Ocular trauma is common in civilian and military populations. Among other injuries, closed globe blunt ocular trauma causes acute disruption of photoreceptor outer segments (commotio retinae) and retinal ganglion cell (RGC) death (traumatic optic neuropathy [TON]), both of which permanently impair vision. Caspase-2-dependent cell death is important and evidenced in models of RGC degeneration. We assessed the role of caspase-2 as a mediator of RGC and photoreceptor death in a rat blunt ocular trauma model.

Methods. Bilateral ballistic closed globe blunt ocular trauma was induced in female Lister-hooded rats and caspase-2 cleavage and localization assessed by Western blotting and immunohistochemistry. Retinal caspase-2 was knocked down by intravitreal injection of caspase-2 small interfering RNA (siCASP2). In retinal sections, RGC survival was assessed by BRN3A-positive cell counts and photoreceptor survival by outer nuclear layer (ONL) thickness, respectively. Retinal function was assessed by electroretinography (ERG).

Results. Raised levels of cleaved caspase-2 were detected in the retina at 5, 24, and 48 hours after injury and localized to RGC but not photoreceptors. Small interfering RNA–mediated caspase-2 knockdown neuroprotected RGC around but not in the center of the injury site. In addition, caspase-2 knockdown increased the amplitude of the ERG photopic negative response (PhNR) at 2 weeks after injury. However, siCASP2 was not protective for photoreceptors, suggesting that photoreceptor degeneration in this model is not mediated by caspase-2.

Conclusions. Caspase-2 mediates death in a proportion of RGC but not photoreceptors at the site of blunt ocular trauma. Thus, intravitreally delivered siCASP2 is a possible therapeutic for the effective treatment of RGC death to prevent TON.

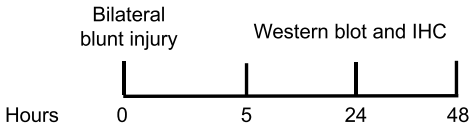
Keywords: traumatic optic neuropathy, commotion retinae, retinal ganglion cells, caspase-2, photoreceptors

Ocular injuries are common, occurring in up to 10% of all military casualties[1] and with a lifetime prevalence of 20% in civilian populations.[2] Traumatic optic neuropathy (TON) occurs with an annual incidence of 1/1,000,000 in the civilian population,[3] but occurs in up to 20% of military eye injuries.[4]

TON is defined as retinal ganglion cell (RGC) death and axon degeneration caused by head or eye injury.[5] RGCs populate the inner retina and their axons form the optic nerve (ON). RGCs are central nervous system (CNS) neurons that lack an endogenous regenerative capacity. Thus, after injury or disease, lost RGCs are not replaced, and the damaged ON does not regenerate, leading to irreversible visual loss.[6–8] The ON may be injured either directly (e.g., penetrating ocular injury, bony fragment damage within the optic canal, and ON sheath hematomas) or indirectly after traumatic head or eye injury (e.g., blunt eye injury and blast).[9–12] Blunt ocular trauma also damages other retinal cells, causing commotio retinae, characterized by photoreceptor degeneration.[13] TON can be studied using animal models replicating blunt and blast ocular injuries[14,15] and ON crush (ONC).[16–18] TON causes permanent visual loss and there are currently no effective treatments to preserve or restore vision.[19,20] After blunt ocular trauma, initiator caspase-9 is activated and initiates localized photoreceptor death, which can be attenuated in the lesion penumbra of animal models by caspase-9 inhibition.[21] However, the mechanisms of the accompanying RGC death in this model have not been defined and no treatment has been shown to neuroprotect RGCs after blunt ocular trauma.[22]

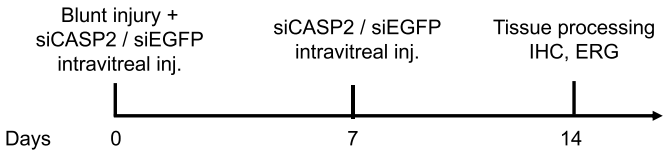
RGCs die by caspase-dependent mechanisms as part of normal development, degenerative disease, and after ON trauma.[23] Caspases are cysteine aspartate proteases that induce apoptosis through initiator and executioner family members. Initiator caspases (2, 8, 9, and 10) activate executioner caspases (3, 6, and 7) through catalytic cleavage of their activation domain.[24–26] Caspase-2 is not part of the canonical intrinsic (caspase-9–mediated) or extrinsic (caspase-8–mediated) apoptotic pathways, is highly evolutionarily conserved and can be activated by DNA damage, heat shock, endoplasmic reticulum stress, and oxidative stress.[27–31] Caspase-2 is activated by cleavage and subsequent dimerization; it is therefore possible to use the presence of cleaved caspase-2 as a marker for its activation.[32]

A *In vivo* protein and IHC studies



Time point	n (retinae)	Measured endpoints
Intact	Western blot: 18 (6 pooled retinae from 3 animals, repeated on 3 separate occasions); IHC: 4	Caspase-2 cleavage and protein localisation
5 hours	Western blot: 18 (6 pooled retinae from 3 animals, repeated on 3 separate occasions); IHC: 4	Caspase-2 cleavage and protein localisation
24 hours	Western blot: 18 (6 pooled retinae from 3 animals, repeated on 3 separate occasions); IHC: 4	Caspase-2 cleavage and protein localisation
48 hours	Western blot: 18 (6 pooled retinae from 3 animals, repeated on 3 separate occasions); IHC: 4	Caspase-2 cleavage and protein localisation

B *In vivo* siCASP2 studies



Treatment	n (eyes)	Measured endpoints
Intact	8	RGC counts, ONL thickness, ERG; all at 14 days
Blunt injury + siCASP2	8	RGC counts, ONL thickness, ERG; all at 14 days
Blunt injury + siEGFP	8	RGC counts, ONL thickness, ERG; all at 14 days

Figure 1. Experimental design of (**A**) in vivo protein and IHC studies and (**B**) in vivo siCASP2 studies.

We have previously shown that RGC death after ONC is caspase-2–mediated. For example, axotomized RGC activate caspase-2, whereas pharmacological inhibition protects ~60% of RGC for up to 21 days after ONC and a chemically modified synthetic short interfering RNA (siRNA) against caspase-2 (siCASP2) protects >95% of RGCs for up to 12 weeks.[33–36] siCASP2 also protects RGC in a mouse optic neuritis model.[37] siCASP2 (also known as QPI-1007) is currently in clinical trials for nonarteritic ischemic optic neuropathy (NAION) (protocol: QRK007 NCT01064505) and acute primary angle-closure glaucoma (protocol: QRK208 NCT01965106) with Quark Pharmaceuticals (Ness Ziona, Israel). Caspase-2 is also activated and induces neuronal degeneration after spinal cord injury (SCI)[38] and in Alzheimer's disease.[39,40]

In this study, we have discriminated between the caspase-9–mediated photoreceptor loss and RGC death by demonstrating that caspase-2 mediates the RGC but not photoreceptor degeneration in a rat blunt ocular injury model, and that siCASP2 structurally and functionally protected RGCs but not photoreceptors.

Materials and Methods

Experimental Design

To determine the role of caspase-2 in RGCs and photoreceptors after blunt eye injury, groups of rats were subjected to unilateral or bilateral blunt eye injury under anaesthesia as a recovery procedure. To determine caspase-2 cleavage and protein localization, Western blotting and immunohistochemistry (IHC) were performed at 5, 24, and 48 hours after injury (Fig. 1A). siCASP2 was used to knockdown caspase-2 and electroretinography (ERG) and cell counting in retinal sections used to determine RGC and photoreceptor survival and restitution of function (Fig. 1B).

Animal Care and Procedures

Animal procedures were licensed by the UK Home Office, approved by the University of Birmingham's Animal Welfare and Ethical Review Committees and conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic

Table. Antibodies Used in IHC and Western Blotting

Antigen	Dilution	Supplier	Catalog No.	To Identify
Caspase-2 (H-19)	1:500 (WB);	Santa Cruz Biotechnology (Santa Cruz, CA, USA)	SC-623	Full-length and cleaved fragments
β-actin	1:10,000 (WB)	Sigma (Poole, UK)	A5441	Loading control
Caspase-2	1:200 (IHC)	Abcam (Cambridge, UK)	AB2251	Full-length caspase-2
BRN3A (C-20)	1:200 (IHC)	Santa Cruz	SC-31984	RGC-specific transcription factor
Donkey anti-goat IgG Alexa Fluor 594	1:400 (IHC)	Invitrogen, (Paisley, UK)	A11058	Goat IgG
Donkey anti-rabbit IgG Alexa Fluor 488	1:400 (IHC)	Invitrogen	A-21206	Rabbit IgG
Donkey anti-rabbit IgG Alexa Fluor 594	1:400 (IHC)	Invitrogen	A-21207	Rabbit IgG
Anti-rabbit IgG, HRP-linked	1:1,000 (WB)	Cell Signaling Technology (Danvers, MA, USA)	7074S	Rabbit IgG
Anti-mouse IgG, HRP-linked	1:1,000 (WB)	Cell Signaling Technology (Danvers, MA, USA)	7076S	Mouse IgG

HRP, horseradish peroxidase; WB, Western blot.

and Vision Research. A total of 68 animals were used in this study (Fig. 1). Female Lister-hooded rats weighing 170 to 200 g were purchased from Charles River Laboratories (Margate, UK), kept on a 12-hour light-dark cycle with a daytime luminance of 80 lux and fed and watered ad libitum. Surgery and ERG recording were performed under inhalational anaesthesia with 3% isoflurane in oxygen (2% for ERG studies). Blunt injury was induced as previously described,[41] using a 0.095-g spherical plastic pellet fired using compressed air to directly impact the inferior scleral surface at a speed of 20 m/s. This creates a localized retinal injury causing 18.4% photoreceptor degeneration by 2 weeks and extensive loss of photoreceptor function, with ERG amplitude reduced to <50% of intact values.[41]

Western Blot

Groups of rats were killed at 5, 24, and 48 hours after bilateral blunt injury, as well as uninjured control animals, by overdose of anaesthetic ($n = 3$ animals per group, six pooled retinae, repeated on three independent occasions; total nine animals per group, 18 retinae). Whole retinae were removed, pooled, and protein extracted in ice-cold lysis buffer (150 mM NaCl, 20 mM Tris, 1 mM EDTA, 0.5 mM EGTA, 1% NP-40, pH 7.4) supplemented with protease inhibitor cocktail (Sigma, Poole, UK), denatured by heating to 90°C for 5 minutes, separated on a Tris-glycine SDS gels with 40 μg protein/lane and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Watford, UK). After probing with primary and secondary antibodies specific protein bands were detected by exposure to photographic film (GE Healthcare, Little Chalfont, UK). Blots were repeated on three independent occasions. Integrated band intensity was measured using the automated gel analysis feature in ImageJ (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) and displayed as % loading control signal (β-actin) ± SEM.

Tissue Preparation for IHC

At 5 and 48 hours after unilateral blunt injury, four rats in each group were killed by intracardiac perfusion with 4% paraformaldehyde (PFA) in PBS. The inferior limbus was marked with a 6.0 Vicryl suture (orientating the injury site). The anterior segment was removed, the injury site identified under light microscopy, and a small section of retina and sclera removed at the 3 and 9 o'clock positions (injury site at 12 o'clock) to allow precise orientation during the embedding process. Retinal cups were cryoprotected in ascending concentrations of sucrose (10%, 20%, 30%) in PBS at 4°C before embedding in optimum cutting temperature medium and storage at −80°C. Sections were cut in a plane parallel to that running between the center of the injury site and the optic disc (Supplementary Fig. 1) at a thickness of 15 μm using a cryostat (Brights Instruments, Huntingdon, UK) and adhered onto Superfrost (Fisher Scientific, Loughborough, UK) coated glass microscope slides and stored at −20°C until required.

Immunohistochemistry

Frozen sections were left to thaw for 20 minutes and washed 3 × 5 minutes in PBS, followed by 20 minutes of permeabilization and nonspecific binding site blocking in 1% Triton X-100 (Sigma) and 3% bovine serum albumin (BSA; Sigma). Tissue sections were incubated overnight at 4°C with primary antibody (Table) in 0.5% Tween-20 and 3% BSA before washing 3 × 5 minutes in PBS and incubating with secondary antibodies (Table) at room temperature. Tissue sections were washed 3 × 5 minutes in PBS then mounted in VectorShield mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Peterborough, UK). Controls with omitted primary antibody were included in each run and these were used to set the background threshold levels for image capture. Sections were viewed under an epi-fluorescent microscope equipped with an AxioCam HRc, controlled using Axiovision Software (all from Zeiss, Hertfordshire, UK).

Antibodies

Primary antibodies used were against full-length caspase-2 for IHC (AB2251; Abcam, Cambridge, UK), full-length and cleaved caspase-2 (H19) for Western blotting and IHC (SC-623, Santa Cruz, CA), BRN3A (C-20) to detect RGCs in IHC (SC-31984; Santa Cruz, CA) and β-actin as a Western blot loading control (A5441; Sigma, Poole, UK). Secondary antibodies were species-specific horseradish peroxidase conjugated for Western blotting (GE Healthcare) and Alexa Fluor 488 or 594-conjugated for IHC (Invitrogen, Paisley, UK). Antibodies used are displayed in the Table.

Intravitreal Injections of siCASP2/siEGFP

siCASP2 (a gift from Quark) was reconstituted in sterile PBS and 5 μL of 4 mg/mL solution delivered by unilateral intravitreal injection immediately after and at 7 days after bilateral blunt ocular injury (Fig. 1, $n = 8$ eyes from eight animals). Contralateral eyes were given control treatment of 5 μL of 4 mg/mL siEGFP (enhanced green fluorescent protein;

gift from Quark) solution. Uninjured rats were culled without intravitreal injections as intact controls ($n = 8$ animals). Rats were culled 14 days after injury and eyes processed in the same way as for IHC.

Electroretinography

Electroretinograms (ERG) were recorded (HMsERG; Ocuscience, Kansas City, MO, USA) at 7 and 14 days after injury and in uninjured controls and were interpreted using ERG View (Ocuscience). Animals were dark-adapted overnight and prepared for ERG under dim red light (>630 nm). Scotopic (dark-adapted) flash ERG were recorded from −2.5 to +1 log units with respect to standard flash in half log unit steps and photopic (light-adapted) flash ERG were recorded with background illumination of 30,000 mcd/m^2 over the same range. DTL fiber (Unimed Electrode Supplies, Farnham, UK) corneal electrodes with pressure-moulded Aclar (Agar Scientific, Stansted, UK) contact lenses were used with needle skin electrodes (Unimed).

ERG traces were analyzed using the manufacturer's semi-automated software ERGView (Ocuscience) and marker position manually verified and adjusted where necessary by a blinded observer.

Assessment of Photoreceptor and RGC Survival

Outer nuclear layer (ONL) thickness was measured in frozen sections as previously described.[21] To account for variability in cell death with respect to distance from the impact site, seven retinal sections per eye were analyzed from each eye: one through the optic disc and center of the impact site (0 μm) and in two sections at 600, 1200, and 1800 μm either side of this plane (see Supplementary Fig. S1). DAPI-stained sections were scanned and the ONL manually segmented in Adobe Photoshop (Adobe Systems, Inc., San Jose, CA, USA) by a masked observer and the ONL area, measured in ImageJ, divided by the length of the retinal segment to give an average ONL thickness. Because the ONL contains only photoreceptor nuclei, this method assesses photoreceptor survival with systematic sampling across the whole retina.

Similarly, RGC survival was measured by counting cells in the ganglion cell layer (GCL) stained for brain-specific homeobox/POU domain protein 3A (BRN3A; an RGC-specific transcription factor and marker)[42,43] across the entire retinal section at the same distances from the center of the injury site (0, 600, 1200, and 1800 μm).

Only the central section is a radial section through the injury site. Sections cut tangentially through the circular zones of injury at the periphery of the injury site will overrepresent cells in these peripheral zones, whereas the single section through the disc and the center of the injury site passes perpendicularly through these zones and so they are proportionally less represented in the overall count for that section.

Statistics

Power calculations performed in G*Power (v. 3.1.4; Kiel University, Kiel, Germany) indicated for Western blot, $n = 3$ animals (six pooled eyes) per time point had 82% power to detect a 1-fold change in protein levels (assuming SD = 20% band intensity); for ERG assessment (more variable than structural measures, therefore less powerful) eight animals had a power of 88% to detect a moderate (f = 0.25) treatment effect (correlation among repeated measures = 0.5 from past data[41]).

All statistical analyses were performed in SPSS 21 (IBM Corp., Armonk, NY, USA). Western blots were analyzed using repeated-measures ANOVA with Tukey post hoc testing. ERG, BRN3A counts, and ONL thickness data were analyzed by fitting a generalized linear model. *T*-tests with Holms Bonferroni correction were applied after generalized linear model for explanatory purposes only, as no meaningful post hoc test was available within subject comparison. Average values are presented as mean ± SEM.

Results

Caspase-2 Is Cleaved in RGC After Blunt Ocular Injury

Western blotting for caspase-2 and its cleaved fragments was performed on pooled retinae after bilateral blunt ocular injury ($n = 3$ animals per experimental group, six pooled retinae, repeated on three independent occasions; total nine animals per group, 18 retinae). Western blotting of whole retinal lysates demonstrated that retinal levels of the cleaved 12-kDa fragment of caspase-2 increased up to 48 hours after injury compared with intact animals (ANOVA $P < 0.01$; Figs. 2A, 2C), though full-length 55-kDa caspase-2 levels remained unchanged (ANOVA $P > 0.05$; Figs. 2A, 2B). Post hoc *t*-test with Tukey's multiple comparisons showed that cleaved caspase-2 significantly increased at 48 hours after blunt ocular trauma compared with intact controls ($P < 0.05$). There was also a significant increase in 12-kDa caspase-2 fragment between 5-hour and 48-hour timepoints ($P < 0.001$) and 24 and 48 hours ($P < 0.05$).

Caspase-2 IHC was performed to demonstrate retinal localization ($n = 4$ animals per timepoint). At 5 and 48 hours after blunt ocular injury, caspase-2 was localized to RGC adjacent to the center of the injury site with increased cytoplasmic caspase-2 expression compared to intact retinae (Fig. 3A). Caspase-2 was also detected in the inner nuclear layer (INL); however, levels were not altered by injury, suggesting that caspase-2 is endogenously expressed in the INL. Immunoreactive caspase-2 was not detected by IHC in the ONL, where photoreceptor cell bodies are present (Fig. 3B).

siCASP2 Protected RGC Adjacent to the Center of the Injury Site But Did Not Protect Photoreceptors

To assess the effects siCASP2 treatment, bilateral blunt ocular injury was performed with unilateral intravitreal injection of siCASP2 and contralateral injection of siEGFP control ($n = 8$ eyes per condition, eight animals); animals were culled at 2 weeks after injury and eyes processed for IHC. Intact animals receiving no injections were also analyzed ($n = 8$ animals). siCASP2 treatment reduced RGC death with an effect that varied by distance from the impact site (generalized linear model $P < 0.01$) (Fig. 4A). For example, BRN3A cell counts as a percentage of intact retinae at 0, 600, 1200, and 1800 μm from the impact site were 73.94% ± 4.51%, 77.73% ± 2.61%, 84.70% ± 3.87%, and 84.64% ± 7.02% in siCASP2-treated eyes compared with 67.39% ± 4.06%, 66.73% ± 2.57%, 82.66% ± 4.59%, and 85.26% ± 4.17% in siEGFP control eyes. Mean counts of BRN3A-positive cells per 1000 μm of retinae for intact, and after bilateral blunt injury with siCASP2 and siEGFP injections are displayed in Figure 4C. Post hoc *t*-testing (with Holms Bonferroni correction for multiple comparison) at each distance, confirmed higher RGC counts immediately peripheral to the impact site (600 μm; $P < 0.05$) in siCASP2-treated compared with siEGFP control retinae, indicating a pro-survival effect of caspase-2 knockdown on RGC in that zone of injury. There was little effect of siCASP2 on RGC survival

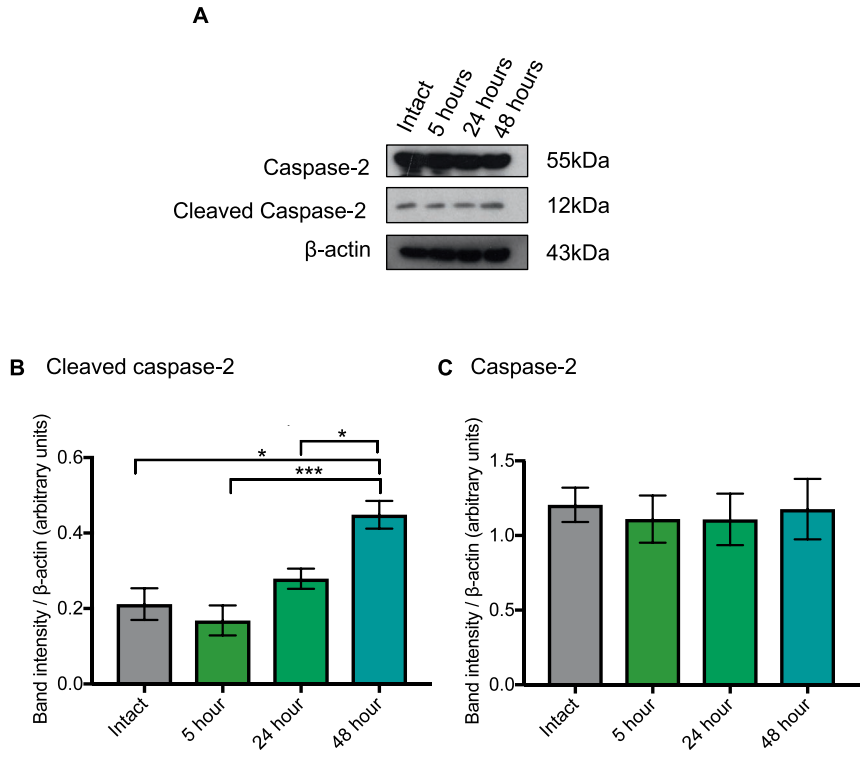


Figure 2. Retinal cleaved caspase-2 is elevated up to 48 hours after blunt ocular injury. Animals received bilateral blunt ocular injury and retinae were collected after 5, 24, and 48 hours. Caspase-2 protein expression was analyzed by Western blot. (**A**) Representative Western blot, showing full-length caspase-2 and cleaved caspase-2. (**B**) Full-length caspase-2 (55 kDa) was not altered after blunt ocular trauma (ANOVA $P < 0.05$). (**C**) Cleaved caspase-2 (12 kDa) was elevated up to 48 hours after blunt ocular trauma (ANOVA $P < 0.01$; post hoc *t*-test with Tukey's multiple comparisons $P < 0.05$). $n = 3$ animals per group with bilateral blunt ocular injury, six pooled retinae, repeated on three independent occasions; total nine animals per group, 18 retinae. *Error bars* represent SEM. $^{*}P < 0.05$, $^{***}P < 0.001$.

central to the impact site (0 μm) and distant to the injury site (1200 and 1800 μm).

To assess the effect of siCASP2 on photoreceptor death in the rat retina, we measured ONL thickness on retinal sections at 600, 1200, and 1800 μm on either side of the impact site as previously described[21] and found no significant effect of siCASP2 treatment on ONL thickness, compared with siEGFP control (Fig. 4B; generalized linear model $P = 0.372$ for an effect of siCASP2, with no significant variation by distance from impact site [$P = 0.942$]). For example, normalized percentages of intact ONL thickness at 0, 600, 1200, and 1800 μm from the impact site were 76.89% ± 9.40%, 74.60% ± 7.02%, 71.75% ± 7.86%, and 70.39% ± 8.16% in siCASP2-treated eyes compared with 73.81% ± 5.54%, 71.98% ± 6.39%, 65.41% ± 5.49%, and 70.5% ± 6.88% in siEGFP control eyes. Mean ONL thickness values at different distances from the center of the impact site in intact, and after bilateral blunt injury with siCASP2 or siEGFP intravitreal injections are displayed in Figure 4D. This suggests that the pro-survival effect of caspase-2 knockdown was specific to RGC.

Caspase-2 Knockdown Induced Significant Functional RGC Neuroprotection After Blunt Ocular Trauma

Bilateral blunt ocular injury was performed with unilateral intravitreal injection of siCASP2 and contralateral injection of siEGFP control ($n = 8$ eyes per condition, eight animals). Scotopic and photopic electroretinography was performed at 2 weeks after injury to assess retinal function after siCASP2 treatment. Intact animals receiving no injections were also analyzed ($n = 8$ animals). There was a reduction in photopic negative (PhNR) amplitude recorded in siEGFP-injected and siCASP2-injected rats compared with those with intact retinae (Fig. 5A), suggesting reduced RGC function after blunt ocular trauma. There was a significant functional protection in PhNR amplitudes in siCASP2- compared with siEGFP-treated animals (Fig. 5A, generalized linear model $P = 0.042$), an effect that did not significantly vary by stimulus intensity across the range tested. Blunt ocular injury also decreased the scotopic a-wave and b-wave and the photopic b-wave amplitudes when compared to intact rats (Figs. 5B–D), but there was no significant effect of siCASP2 treatment on these waveforms (Figs. 5B–D; scotopic a, $P = 0.145$; scotopic b, $P = 0.503$; photopic b, $P = 0.889$), suggesting a specific effect on RGC function.

Discussion

Our model of blunt ocular trauma causes commotio retinae and TON.[41] It is established that photoreceptors structurally and functionally degenerate in this model and there is a reduction in the number of cells in the GCL at the center of the impact site, with less death toward the periphery.[41] Previous studies have shown that photoreceptor death is mediated by caspase-9,[21] but the mechanisms of RGC death in this model have not been previously investigated. Here we show that caspase-2 is immunolocalized to injured RGC and that levels of the active form, cleaved caspase-2, increased over 48 hours after blunt ocular trauma. Activated caspase-2 induces RGC death in diverse models of RGC degeneration, including ONC (direct TON),[33–35] glaucoma,

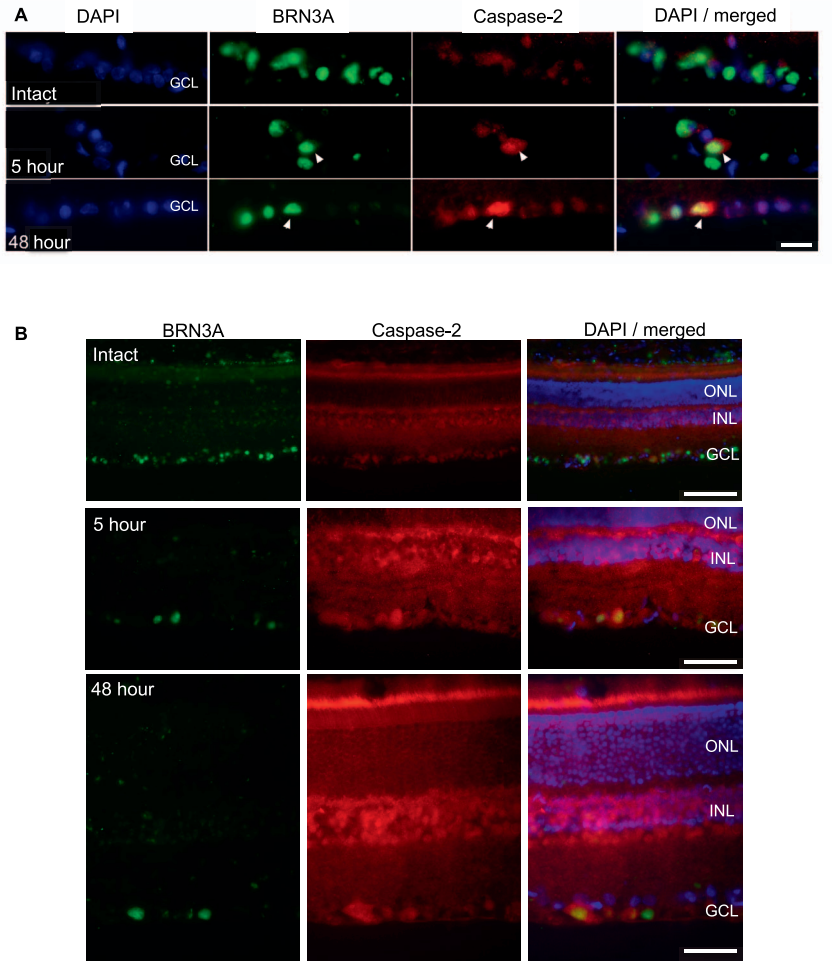


Figure 3. Caspase-2 (*red*) is localized to RGCs (*green*) but not to photoreceptors. (**A**) Caspase-2 (*red*) is localized to BRN3A- (*green*, an RGC-specific transcription factor) positive cells in the GCL. Caspase-2 is present at 5 and 48 hours after injury, but is less present in intact retinae. (**B**) Caspase-2 is not localized to photoreceptor cell bodies in the outer nuclear layer (ONL). There is caspase-2 immunostaining in the INL, but this is not altered at 5 and 48 hours after blunt ocular injury. Nuclei were counterstained with DAPI (*blue*). (**A**) *Scale bar*: 20 μm. (**B**) Intact, *scale bar*: 100 μm; 5-hour and 48-hour *scale bar*: 50 μm. $n = 4$ animals per group.

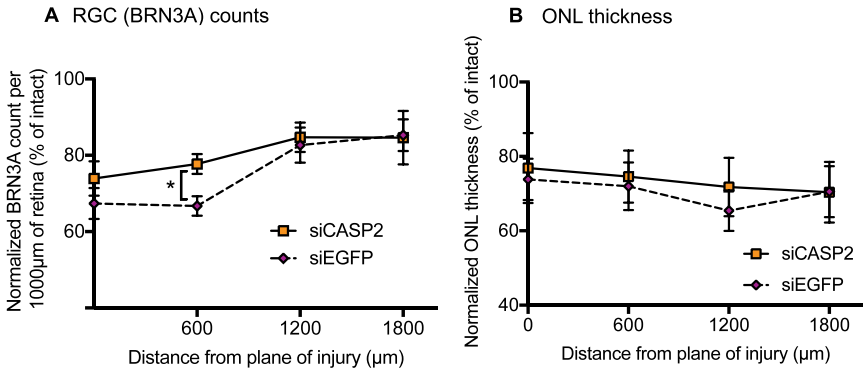
and optic neuritis.[37] Inhibition of caspase-2 protects >95% of RGCs from death after ONC.[33,35] Our study with siCASP2 suggests that RGC death in TON induced by blunt ocular trauma is caspase-2-dependent and suggests a new therapeutic treatment for this condition.

In contrast, caspase-2 did not immunolocalize to photoreceptors after blunt ocular trauma and caspase-2 knockdown using siCASP2 did not affect photoreceptor survival, suggesting that photoreceptor death is independent of caspase-2 and that other mechanisms are responsible for photoreceptor death; for example, caspase-9–dependent mechanisms.[21]

In models of ocular blast injury, caspase-1 and other cell death molecules, including receptor interacting protein kinase (RIPK) 1 and 3, are localized to Müller cells and the inner nuclear and inner plexiform layers,[44] which suggests different cell death signaling pathways in an ocular blast injury model. However, caspase-2 is consistently implicated in RGC death after various types of insults.[33,35–37] In agreement with our assertion that caspase-2 is an important cell death mediator in RGC, caspase-2 is also implicated in the neuronal death that occurs in some neurodegenerative diseases, including models of Alzheimer's disease, where caspase-2 cleavage of tau impairs cognitive and synaptic function and downregulation of caspase-2 restores long-term memory,[40] as well as in β-amyloid–induced neurodegeneration in vitro.[39]

IHC is often used to demonstrate caspase activation using antibodies against full-length enzymes, despite them not showing caspase cleavage or activation. Another way to show caspase activity is through the use of pharmacologic inhibitors of caspases, such as z-VAD-fmk. However, the active sites of these pharmacological inhibitors are nonspecific and have cross-reactivity with other caspases and noncaspase targets, such as calpains.[45–48] In addition, pharmacological inhibition of caspase-2 is not as efficient as RNA interference using siCASP2 at attenuating RGC loss, protecting only 60% of RGC from death at 21 days after ONC,[36] compared with >95% RGC protection achieved by siCASP2.[33,35] By contrast, our use of siRNA knockdown is highly specific to caspase-2, without activating nonspecific innate immunity[33] and our study of caspase-2 cleavage products ensures that cleaved caspase-2 is present.

Previous studies have extensively studied the effects of siCASP2 in vitro and in vivo after ONC.[33] siCASP2 shows a significant knockdown of caspase-2 mRNA in vitro using quantitative PCR; with a >80% knockdown in human HeLa cells and an approximately 65% knockdown in rat PC12 cells. Also, in vivo there was an approximately 50% knockdown of



C

	Mean number of BRN3A cells per 1000µm of retinae ± SEM			
Treatment	0µm	600µm	1200µm	1800µm
Intact	56.9 ± 2.19	59.9 ± 1.85	57.5 ± 2.97	63.1 ± 5.55
Blunt + siCASP2	42.1 ± 2.57	46.5 ± 1.56	48.7 ± 2.22	53.4 ± 4.43
Blunt + siEGFP	38.4 ± 2.31	39.9 ± 1.54	47.5 ± 2.64	53.8 ± 2.63

D

	Mean number of pixels of ONL thickness ± SEM			
Treatment	0µm	600µm	1200µm	1800µm
Intact	44.6 ± 0.86	46.2 ± 1.77	54.8 ± 3.65	65.6 ± 4.34
Blunt + siCASP2	34.3 ± 4.21	34.5 ± 3.24	39.4 ± 4.31	46.2 ± 5.35
Blunt + siEGFP	32.9 ± 2.47	33.3 ± 2.96	35.9 ± 3.01	46.3 ± 5.52

Figure 4. siCASP2 prevents RGC death but does not protect photoreceptors. (**A**) BRN3A- (RGC-specific transcription factor) positive RGCs were counted across the entire retinae and reported as a percentage of intact BRN3A counts per 1000 µm of retinae. Unilateral intravitreal injection of siCASP2 promoted RGC survival (generalized linear model $P < 0.05$), with an effect at 600 µm peripheral to the center of the injury site (*t*-test with Holms Bonferroni correction for multiple comparison at 600 µm $P < 0.05$). At the center of the injury site (0 µm), RGC death is not prevented by siCASP2. In the distant periphery (1200 µm, 1800 µm), there is less RGC degeneration and no significant effect of siCASP2. (**B**) Photoreceptors are not protected by siCASP2 (generalized linear model $P > 0.05$). ONL thickness is reduced after blunt ocular injury and siCASP2 does not improve ONL thickness compared with siEGFP control. Experiments represent $n = 8$ animals per group; eight animals received bilateral blunt ocular trauma with unilateral siCASP2 intravitreal injection and contralateral siEGFP control injection. $n = 8$ intact animals were used. Error bars represent SEM. (**C**, **D**) Raw values in intact animals and after blunt ocular trauma with siCASP2 or siEGFP treatment for (**C**) mean BRN3A-positive cells per 1000 µm of retinae and (**D**) mean ONL thickness (pixel count).

caspase-2 mRNA in Thy1.1 isolated RGC after intravitreal injection of siCASP2 compared with siEGFP-injected controls; however, these differences did not reach statistical significance. It also has specific RNAi-mediated caspase-2 mRNA cleavage, as shown through RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) experiments, which show the detection of caspase-2 mRNA-specific cleavage product.[33] Further, siCASP2 has chemical modifications in both sense and antisense strands, which prevent its degradation by vitreal and serum nucleases.[33] siCASP2 does not activate the innate immune system, shown through a lack of in vivo interferon responses and in vitro cytokine production[33] and has low-risk systemic toxicity.[49] Together, these data suggest that siCASP2 knocks down caspase-2 in vitro and in vivo, does not induce an inflammatory response, and has specific RNA interference-mediated cleavage of caspase-2.

After blunt ocular trauma, siCASP2 protected RGC from death adjacent to the impact site, suggesting that this is where the highest proportion of RGCs undergoing caspase-2–dependent cell death are found. In contrast, RGC central to the impact site, where more severe injury presumably predisposes cells to necrosis, were less susceptible to modulation by altered caspase-2 activity. Only a proportion of degenerating RGCs in the immediate periphery of the lesion site were protected by siCASP2, suggesting that the remaining proportion die by alternative cell death mechanisms (such as necroptosis or pyroptosis) or unregulated necrosis, which remains to be elucidated. At greater distances from the impact site, less RGC degeneration occurs, so no effect of siCASP2 was seen.

The differential protection of retinal neurons by siCASP2 was also reflected by preservation of their function. The scotopic a-wave is the first negative deflection of the flash ERG wave and is predominantly caused by photoreceptor hyperpolarization. In rats, the scotopic a-wave amplitude represents rod function. siCASP2 did improve the scotopic a-wave, which

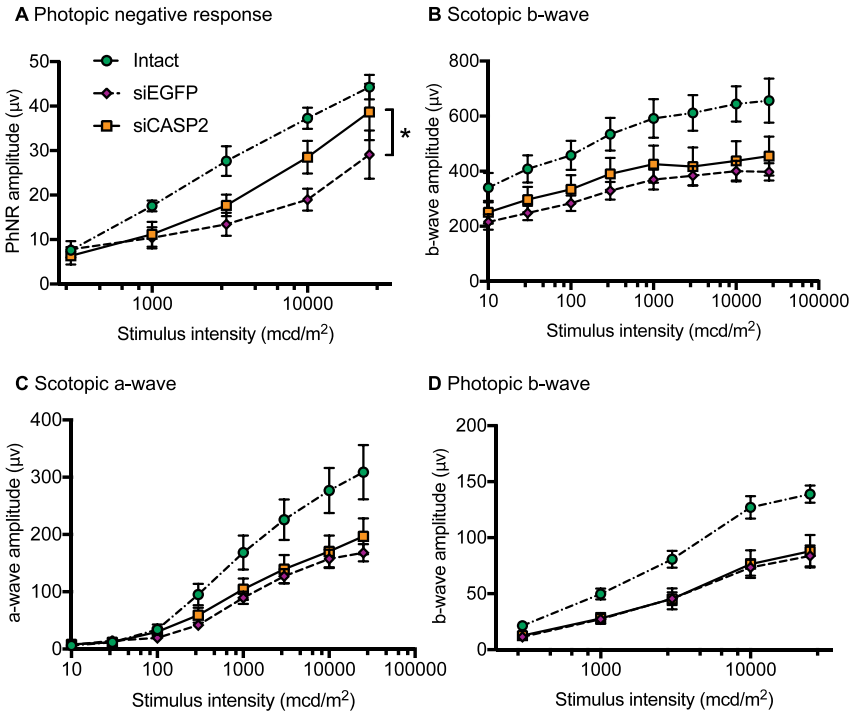


Figure 5. ERG shows reduced retinal functional at 14 days after blunt ocular injury, with a protective effect of siCASP2 on RGC function only. (**A**) There is a reduced PhNR amplitude after blunt ocular injury. PhNR amplitude is partly rescued by siCASP2 compared with siEGFP control (generalized liner model $P = 0.042$). (**B–D**) Amplitudes of scotopic b-wave, scotopic a-wave, and photopic b-wave are reduced after blunt ocular trauma, but are not rescued by siCASP2. $n = 8$ animals; eight animals received bilateral blunt ocular trauma with unilateral siCASP2 intravitreal injection and contralateral siEGFP control injection. Eight intact animals were also analyzed. *Error bars* represent SEM.

suggests that rod photoreceptor function was not protected by caspase-2 knockdown. Under photopic conditions, rod photoreceptors are bleached, meaning the photopic a-wave is cone-mediated. In rats, the photopic a-wave amplitude is small; therefore, variations in b-wave amplitude were used as a downstream measure of photoreceptor function, which is also dependent on bipolar cell function. Under both scotopic and photopic conditions, a-wave and b-wave amplitudes were reduced after blunt ocular injury, but showed a lack of functional improvement after siCASP2 treatment. These results are consistent with the lack of effect of siCASP2 on ONL thickness, suggesting that siCASP2 has no protective effect on photoreceptor structure or function.

The photopic negative response (PhNR) is a downstream measure of retinal function, dependent on activity in first and second order neurons (photoreceptors and bipolar cells).[50] Thus, a change in PhNR may be caused by changes in either RGC function or upstream cells such as photoreceptors. The PhNR is commonly used to assess RGC function; it is reduced in experimental and human glaucoma[51] and is correlated with RGC loss in ON transection.[52] Despite rat photopic responses being heavily amacrine cell-dependent,[53] a significant proportion of the response is also RGC-dependent.[52] Because siCASP2 treatment did not affect photopic b-wave amplitude, any change in PhNR was derived from effects downstream of ON-bipolar cells. siCASP2 increased PhNR amplitude at 14 days after injury compared with siEGFP controls, suggesting that surviving RGCs, in which caspase-2–dependent cell death was prevented, remain potentially viable and functional. The elevated PhNR amplitude could also reflect increased electrical activity in RGCs that are dysfunctional but not dead, which would be consistent with the >50% reduction in ERG amplitude after injury,[41] despite <20% photoreceptor degeneration, and the preservation of the PhNR amplitude in siCASP2-injected eyes at near-normal levels. Nonetheless, the improved PhNR amplitude raises the possibility that siCASP2 induces functional RGC neuroprotection after blunt ocular trauma.

In conclusion, we show that in blunt ocular trauma, caspase-2 mediates degeneration of a proportion of compromised RGC and that siCASP2 provides functional neuroprotection to RGC peripheral to the injury site. By contrast, caspase-2 is not localized to photoreceptors and siCASP2-mediated caspase-2 knockdown does not structurally or functionally protect photoreceptors after retinal injury, suggesting that caspase-2 is active exclusively in RGCs. Observations that caspase-2 is activated in RGCs and neurons compromised in other neurodegenerative diseases and in trauma[33,35,38–40] imply that caspase-2–dependent signaling pathways may be common among CNS diseases and that siCASP2, if successful in clinical trials, has the potential to be a widely transferable therapy.

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